Malays. Appl. Biol. (2015) 44(1): 101–106

ECTOPIC EXPRESSION OF *OPKN1* GENE IN *N. benthamiana* ALTER FLOWER SIZE AND DELAY FLOWERING TIME

MOHD WAZNUL ADLY, M.Z.[1,2], ZURAIDA, A.B.[2], WEE, C.Y.[2], ALIZAH, Z.[2], NORLIZA, A.B.[2] and CHE RADZIAH, C.M.Z.[1*]

[1]School of Biosciences & Biotechnology, Faculty of Science & Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[2]Molecular Biology & Genetic Engineering Programme, Biotechnology Research Centre, Malaysian Agriculture, Research & Development Institute, 43400 Serdang, Selangor, Malaysia
[*]E-mail: cradziah@ukm.edu.my

ABSTRACT

Oil Palm Knotted-like 1(*OPKN1*) gene is a type of homeobox gene that was previously isolated from cDNA library of oil palm's suspension cell culture. Based on semi-quantitative RT-PCR, northern blotting and *in situ* hybridization analysis, the *OPKN1* gene is active in flower meristem suggesting its involvement in flowering phase. Semi-quantitative RT-PCR analysis indicated that *OPKN1* gene is active in both male and female flowers and also in abnormal flower suggesting its role in flower morphogenesis process. To further validate the function of this gene, the overexpression transformation vector of *OPKN1* gene driven by CaMV 35S constitutive promoter was constructed. The constructed vector was transformed into plant model systems *Nicotiana benthamiana* via *Agrobacterium tumefaciens*. Four transgenics lines of *N. benthamiana* were successfully obtained and analysis of quantitative Real-Time PCR shows that *OPKN1* gene was highly overexpressed in all transgenic lines. Overexpression of *OPKN1* gene in *N. benthamiana* transformant has caused reduction in *N. benthamiana*'s flower size and flowering time was also observed to be delayed. Based on this finding, it was suggested that *OPKN1* may be involved in flower development and morphogenesis process.

Key words: Knotted gene, oil palm, OPKN1, gene expression, *N. benthamiana*

INTRODUCTION

Knotted-like homebox (*KNOX*) gene is a gene which is classified in big superfamily of *Three Amino Loop Extention* (*TALE*). This gene contains conserved homedomain and *ELK* and also has a conserved *KNOX* motif known as a *MEINOX* that functions in protein-protein interaction. The *KNOX* gene also contains helix motif at *ELK* domain which is also reported to involve in protein-protein interaction. The *TALE* structure presents in plant *KNOX* gene was also found in animal and fungi suggesting it derivation from the same ancestor (Mukherjee *et al.,* 2009). In plants, *KNOX* is divided into two classes which are class 1 and class 2 depending on the similarity of residue on homeodomain and also intron position (Chan *et al.,* 1998). The *KNOX* gene was also grouped based on gene expression pattern and also the similarity of gene sequence (Kerstetter *et al.,* 1994). Generally, class 1 *KNOX* gene is mainly expressed at apical meristems and at embryo stage. Previously, it was reported that most of class 1 *KNOX* was expressed in plant meristem but not at the lateral organ and it is involved in maintenance of plant meristem, leaf development, senescence process, hormone pathways and also involve in flowering structure. In other hand, class 2 *KNOX* gene show wider range of gene expression pattern in plant (Smith *et al.,* 1992).

Research on *Zea mays Knotted-like 1* (*ZMKn1*) gene show that it is not highly expressed during maize leaf development but it is active at the apical part of maize flower and vegetative. Research conducted on *ZMKn1* gene suggested that plant homeobox has important role in cell fate determination. Mutation of *ZMKn1* in maize has caused shootless phenotype during cotyledon development (Vollbrecht *et al.,* 2000). Another example of class 1 *KNOX* gene is *SHOOTMERISTEMLESS* (*STM*) gene in *Arabidopsis*. *STM* gene have a close relationship with *ZmKn1* gene based on the similarity of protein sequence and gene expression pattern which was found expressed at whole part of *Arabidopsis* meristem (Vollbrecht

* To whom correspondence should be addressed.

et al., 2000). Long *et al.* (1996) also reported that *Arabidopsis STM* gene is a type of class 1 *KNOX* gene which is essential for shoot apical meristem formation during embryogenesis.

Oil Palm Knotted Homebox or *OPKN1* gene is a type of homeobox gene isolated from cDNA library of oil palm suspension cell culture. This 1310 bp gene was found to have a lot of similarity with class 1 protein of knotted-like 1. Based on BLASTX analysis, homeodomain sequence of *OPKN1* gene has close similarity with maize knotted-like 1 sequence suggesting it classified under knotted-like 1 group. Che Radziah (2005) have reported that *OPKN1* gene was highly expressed in oil palm vegetative and flower meristem suggesting it function in vegetative and reproductive phase for oil palm growth. In addition, the *OPKN1* gene was also expressed in oil palm's embryogenic culture which is indicate that it involvement in development of oil palm's embryogenesis. However, distribution of *OPKN1* activity in oil palm vegetative meristem is differing from others species based on the specific localization of *OPKN1* expression in vegetative meristem. Other difference of *OPKN1* gene is the expression was highly detected during leaf primordial development. This expression pattern indicated that the involvement of *OPKN1* in leaf development especially during changes in leaf morphology from lanceolat to bifurcate and lastly to pinnate in oil palm frond. Overall, *OPKN1* do not have specific activity in oil palm part since it was expressed in many phase of oil palm's development. Since *OPKN1* poses a few different pattern of activity, further research is needed to reveal the *OPKN1* gene function in more details. Therefore, further study of *OPKN1* gene using plant model systems in needed to have more understanding on *OPKN1* gene especially in oil palm development.

MATERIALS & METHODS

Construction of *OPKN1* expression vector

OPKN1 gene was cloned into pMR104a vector flanked by CaMV 35S promoter and NOS terminator. The *OPKN1* expression cassette was digested and then ligated into pCAMBIA 1305.2 vector. This vector known as pEx*OPKN1* vector was transformed into *Agrobacterium tumefaciens* strain LBA 4404 and was further used for *Agrobacterium*-mediated transformation of *OPKN1* gene into *N. benthamiana*.

Agrobacterium-mediated transformation of *OPKN1* gene into *N. benthamiana*

Transformation of *OPKN1* gene into *N. benthamiana* via *Agrobacterium* was based on method reported by Hanania *et al.* (2009). *N. benthamiana* leaf disks infected with *A. tumefaciens* carrying pEx*OPKN1* plasmid were cultured in MS media supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. The media was also contains 250 mg/L carbenicilin to eliminate *Agrobacterium* growth and 40 mg/L hygromycin for selection of transformant *N. benthamiana* cells. The infected *N. benthamiana* leaf disks were cultured for 3 weeks and were sub-cultured every 3 weeks. Any plantlet generated from leaf disk was transferred to flask jar containing MSO with 40 mg/L Hygromycin. Transformant *N. benthamiana* with good rooting condition was transferred to soil.

Molecular screening of transformant *N. benthamiana*

Genomic DNA of transformant *N. benthamiana* was isolated using modified CTAB method (Doyle & Doyle 1987). Isolated genomic DNA was screened by PCR amplification using Go Taq® polymerase (Promega, USA) for detection of *OPKN1* gene (*OP1F2: 5' AGTATCCATCCGGTAATCTCGC 3', OP1R2: 5' CTTCTGTCAAGCCCTGTCATC 3'*), *GUS* gene (*GUSF:5' CGCCGATGCAGATATTCGTA 3', GUSR: 5' ATTAATGCGTGGTCGTGCAC 3'*) and *HPT* gene *(HPTF: 5'ACAGCGTCTCCGACCTGATGCA 3', HPTR: 5' AGTCAATGACCGTGTTATGCG 3'*) together with amplification of endogenous *NAD5* gene (NAD5F: 5' TAGCCCGACCGTAGTGATGTTAA 3', NAD5R: 5' ATCACCGAACCTGCACTC AGGAA 3') as an internal control.

Gene expression profiling of *OPKN1* gene in transformant *N. benthamiana*

Total RNA from transformant *N. benthamiana* was isolated using RNAzol® RT reagent (Molecular Research Center, Inc., USA). RNA with good purity and quality was converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany). The cDNA was diluted 10 times and was used as a template for analysis of *OPKN1* gene expression using qPCR comparative $\Delta\Delta_{CP}$ Method with normalization of geometric averaging of multiple internal control genes (Vandesompele *et al.,* 2002). The qPCR analysis reaction was performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA) and the reaction using SensiFAST SYBR Hi-ROX Kit (Bioline, UK) based on manufacture recommendation. The expression of *OPKN1* genes (*KN2F: 5' ATCCTACTGCCGGGTTCTG 3', KN2R: 5' GAGGTGGTTCGTCCTTTGC 3'*) in transformant lines were compared to the wild type *N. benthamiana* and two housekeeping genes which are *EF1A* (EF1AF: 5' GATTGGTGGTATTGGAACTGTC 3', EF1AR: 5' AGCTTCGTGGTGCATCTC 3' and *ACT* (ACTF: 5' TTGAAGCTATTTGCTGATAGAGAGAC 3', ACTR: 5' TGGGCATTTTTATCTTGGATG 3') were used as internal control for normalization.

Phenotypic analysis of transformant *N. benthamiana*

Phenotypic comparison between *N. benthamiana* transformant and wild type was observed between 7 to 75 days after transferred into potted soil. The characterization of transformant plant was identified and measured for each line with wild type *N. benthamiana* as a comparative subject for evaluation of phenotypic characteristics. Differentiation of phenotypic characteristics between *N. benthamiana* transformant and wild type were further analysed using Microsoft Excel in order to determine the differences between transformant and wild type.

RESULTS & DISCUSSION

The *OPKN1* expression vector was successfully constructed by incorporating CaMV 35S promoter and NOS terminator flanking in between *OPKN1* gene sequence. The *OPKN1* expression cassette was then ligated into pCAMBIA 1305.2 to yield approximately 14.4 kb *OPKN1* transformation vector (pEx*OPKN1*) (Fig. 1). pEx*OPKN1* transformation vector which carrying *OPKN1* gene, *GUSA* reporter gene and *HPT* selection gene was successfully transformed into *A. tumefaciens* LBA 4404.

Transformation of *OPKN1* gene via *A. tumefaciens* was conducted on *N. benthamiana's* leaf disk and selection of transforman lines was successfully carried out with 4 lines of transformant *N. benthamiana* were regenerated (Fig. 2). The transformant plantlets of *N. benthamiana* were induced for rooting process and the plantlet successfully transferred to potted soil. In order to determine the successful *OPKN1* gene introduced into transformant *N. benthamiana*, molecular screening by PCR amplification was performed on all transformant lines. The transgenes introduced by *A. tumefaciens* infection consisting of *OPKN1*, *GUSA*

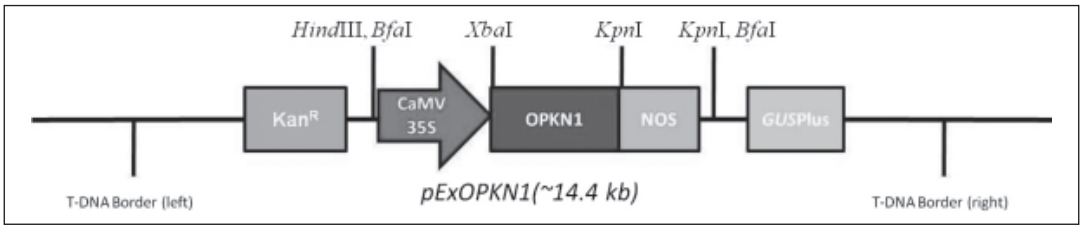

Fig. 1. pEx*OPKN1* plasmid with *OPKN1* gene expression cassette driven by CaMV 35S promoter with pCAMBIA 1305.2 as a plasmid backbone.

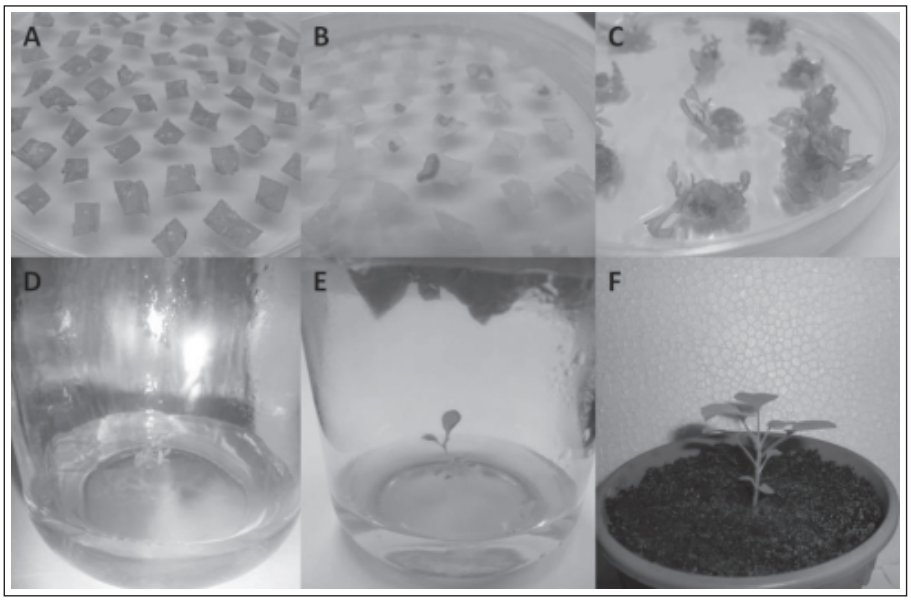

Fig. 2. *In vitro* selection and regeneration of transformant *N. benthamiana*. A) Leaf disks after infection with *A. tumefaciens*: pExOPKN1. B) Transformed leaf disks after 1 month in hygromycin selection media. C: 2 months of transformed leaf disks in hygromycin selection media. D) Untransformed (wild type) *N. benthamiana* plantlet in selection regeneration media. E) Transformed *N. benthamiana* plantlet in in selection regeneration media. F) Transformant *N. benthamiana* in potted soil.

and *HPT* genes were successfully detected in all transformant lines (Fig. 3). 801 bp of *OPKN1* PCR product was detected in genomic DNA sample of *N. benthamiana* transformant lines, T1, T2, T3 and T4. Together with PCR amplification of *OPKN1* gene, PCR amplification of *GUSA* and *HPT* genes with PCR product 750 bp and 594 bp respectively in T1, T2, T3 and T4 transformant lines were also positively detected. However, no amplification for *OPKN1*, *GUSA* and *HPT* genes was detected in wild type *N. benthamiana* which act as a negative control plant.

Gene expression analysis of *OPKN1* gene using quantitative Real-Time PCR (qPCR) in all transformant lines show that *OPKN1* gene was over-expressed in all lines. The expression of *OPKN1* gene in transformant *N. benthamiana* was 202.91, 94.27, 143.35 and 98.72 fold overexpressed for lines T1, T2, T3 and T4 respectively when compared to wild type plants (Fig. 4). This result indicated that the presence of *OPKN1* transcript in transformant plant suggesting that the introduced *OPKN1* gene cassette is transcribed to mRNA. Nevertheless, no similarity of the *OPKN1* expression level between transformant lines and this difference suggesting the transgene may have different copy number and the integration site of *OPKN1* gene for each line may be different in each *N. benthamiana* lines.

Four line of transformant *N. benthamiana* gave similar characteristic of flower morphology which the size of flower in transformant plant is smaller when compared to the wild type plant (Fig. 5 & Table 1). The average length of transformant *N. benthamiana* flower is 3.4 ± 0.2 cm, 3.2 ± 0.2 cm,

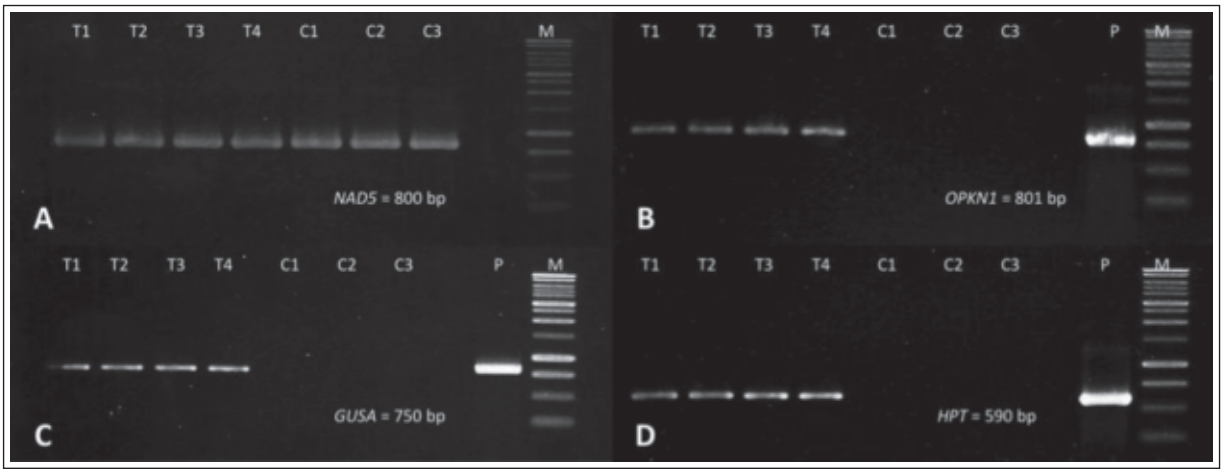

Fig. 3. Molecular screening of transformant *N. benthamiana* by PCR amplification of *OPKN1*, GUSA and HPT genes. A) PCR amplification of endogenous *NAD5* gene. B) PCR amplification of *OPKN1* gene. C) PCR amplification of *GUSA* gene. D) PCR amplification of *HPT* gene. T1, T2, T3 & T4: Transformant lines of *N. benthamiana* transformed with *OPKN1* genes, C1, C2 & C3: Wild type *N. benthamiana*, M: 1 kb ladder (Promega).

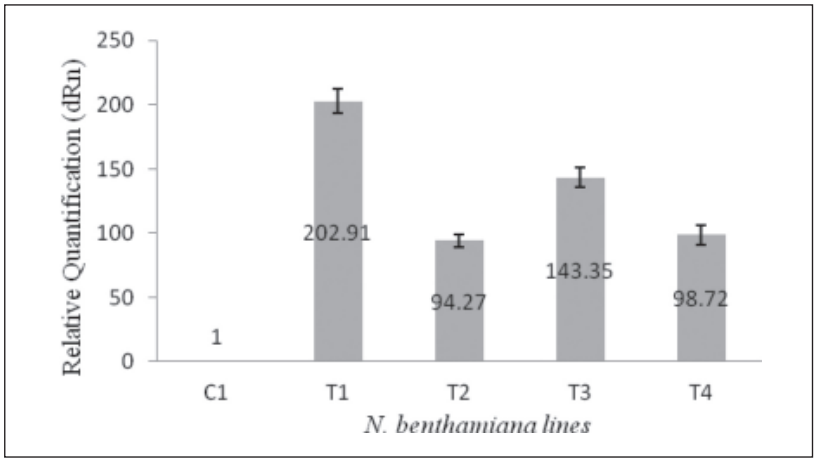

Fig. 4. Relative quantification of *OPKN1* transgene expression in transformant *N. benthamiana* lines T1, T2, T3 & T4 when compared to wild type *N. benthamiana* after reference gene normalization. Differential expression was shown in fold-change number when compared to control (wild type).

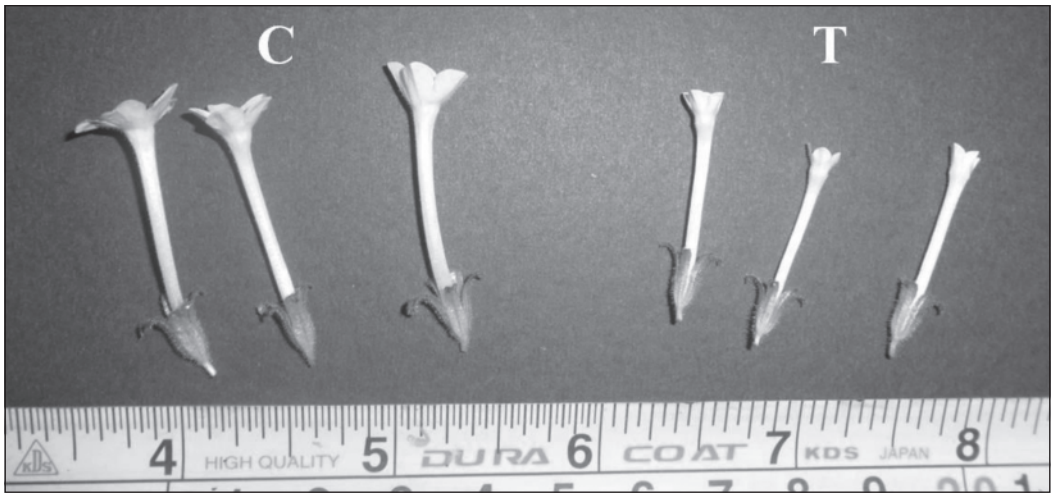

Fig. 5. Morphology comparison of transformant and wild type *N. benthamiana* flower. C: Flowers from wild type *N. benthamiana*. T: Flowers from transformant *N. benthamiana* transformed with *OPKN1* gene.

Table 1. Phenotypic characterization of transformant and control *N. benthamiana* based on flower size and flowering time

Lines	Length of flower (cm)	Flowering time (day)
Transformant T1	3.4 ± 0.2	67
Transformant T2	3.2 ± 0.2	65
Transformant T3	3.3 ± 0.1	73
Transformant T4	3.2 ± 0.2	71
Wild type (control)	4.2 ± 0.2	59 ± 3

3.3 ± 0.1 cm and 3.2 ± 0.2 cm for lines T1, T2, T3 and T4 respectively when compared to the wild type *N. benthamiana* with average flower length is 4.2 ± 0.2 cm. Furthermore, the transformant lines were also detected to delays flowering when compared to the wild type plants. The average period for wild type *N. benthamiana* to produce flower is 59 ± 3.0 days but for *OPKN1* transformant lines, the duration to produce flower are 67, 65, 73 and 71 days for lines T1, T2, T3 and T4 respectively.

Productions of smaller sized flower in transformant *N. benthamiana* are most likely caused by the over-expression of *OPKN1* gene which was transformed into *N. benthamiana*. The previous study reported on lettuce (*Lactuca sativa*) over-expressed with *Arabidopsis kn1* (*KNAT1*) gene has produced dwarf flower with normal morphology but flowers was produced in lesser amount in transgenic lettuce (Frugis *et al*., 2001). Ectopic expression of rice *kn1* (*OSH1*) gene in *Arabidopsis* was also produced dwarf flower in *Arabidopsis* but the morphology of flowers was abnormal when compared to wild type plant (Matsuoka *et al.,* 1993). A delay flowering in transformant *N. benthamiana* suggesting that the *OPKN1* gene delays the plant to produce flower and this is in agreement with result obtained from over-expressing maize *kn1* which caused delayed flowering in transformed maize (Bolduc and Hake, 2009).

It is suggested that high cytokinin content caused reduction of flower size and delayed flowering time in transformant *N. benthamiana* which over-expressing *OPKN1* gene. Generally, cytokinin involved in plant cell division and high content of cytokinin was reported in transgenic plant that over-expressed *kn1* gene (Ori *et al.,* 1999). Ori *et al.* (1999) also reported that the characteristics of tobacco over-expressed *kn1* gene were similar to the transgenic tobacco over-expressed the gene for the cytokinin production, isopentenyl transferase gene (*IPT*). The evidence of *kn1* gene induced cytokinin content was also revealed by Sakamoto *et al.* (2006) on the research of *kn1* protein causing the activation of isopentenyl transferase (*IPT7*) transcription. Instead of that, addition of high dosage of cytokinin caused reduction in bulb's flower size, pedicel length, number of flower and also delaying the flowering time (Pobudkiewicz, 2008). Research conducted on *Arabidopsis thaliana* also has revealed that the accumulation of high cytokinin content through activation of genes related to cytokinin caused delayed flowering time (Igari *et al.,* 2008).

High level of exo-cytokinin blocked the increase in length and cell number of the meristematic cells and also elongation of meristematic zone of *Arabidopsis* suggesting that the balancing of auxin-cytokinin hormones may control the size of the meristem (Beemster and Baskin, 2000). Therefore, the size and the number of floral meristem are proportional to the size of

flower being produced. Based on reduction of flower size and delayed flowering time in transformant *N. benthamiana*, it is suggested that the over-expression of *OPKN1* gene increase the production of cytokinin in transformant *N. benthamiana*. However, further analysis need to be carried out in order to determine the effect of *OPKN1* overexpression on level of cytokinin content and also the possibility to study the expression profile of genes related to cytokinin production in transformant *N. benthamiana*.

ACKNOWLEDGEMENTS

The authors would like to thanks Malaysian Agriculture, Research & Development Institute (MARDI) and National University of Malaysia (UKM) for providing research materials and facilities.

REFERENCES

Beemster, G.T. & Baskin, T.I. 2000. *STUNTED PLANT 1* mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of *Arabidopsis*. *Plant Physiology,* **124(4)**: 1718-1727.

Bolduc, N. & Hake, S. 2009. The maize transcription factor knotted1 directly regulates the gibberellin catabolism gene *ga2ox1*. *Plant Cell,* **21(6)**: 1647-1658.

Chan, R.L., Gago, G.M., Palena, C.M. & Gonzalez, D.H. 1998. Homeoboxes in plant development. *Biochimica et Biophysica Acta* (BBA) – *Gene Structure and Expression,* **1442(1)**: 1-19.

Che Radziah Che Mohd Zain. 2005. Isolation and characterization of a full-length knotted gene from oil palm (*Elaeis guineensis* Jacq.). PhD thesis, Universiti Putra Malaysia, Serdang.

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin,* **19**: 11-15.

Frugis, G., Giannino, D., Mele, G., Nicolodi, C., Chiappetta, A., Bitonti, M.B., Innocenti, A.M., Dewitte, W., Van Onckelen, H. & Mariotti, D. 2001. Overexpression of *KNAT1* in lettuce shifts leaf determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins. *Plant Physiology,* **126(4)**: 1370-1380.

Hanania, U., Velcheva, M., Sahar, N., Flaishman, M., Or, E., Dgani, O. & Perl, A. 2009. Suppression and overexpression of ubiquitin extension protein s27a affects cell proliferation and in vitro regeneration in *Nicotiana benthamiana*. *Plant Science,* **176(4)**: 566-574.

Igari, K., Endo, S., Hibara, K., Aida, M., Sakakibara, H., Kawasaki, T. & Tasaka, M. 2008. Constitutive activation of a CC-NB-LRR protein alters morphogenesis through the cytokinin pathway in Arabidopsis. *The Plalnt Journal,* **55(1)**: 14-27.

Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J. & Hake, S. 1994. Sequence analysis and expression patterns divide the maize *knotted*1-like homeobox genes into two classes. *Plant Cell,* **6(12)**: 1877-1887.

Long, J.A., Moan, E.I., Medford, J.I. & Barton, M.K. 1996. A Member of the knotted class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. *Nature,* **379(6560)**: 66-69.

Matsuoka, M., Ichikawa, H., Saito, A., Tada, Y., Fujimura, T. & Kano-Murakami, Y. 1993. Expression of a rice homeobox gene causes altered morphology of transgenic plants. *Plant Cell,* **5(9)**: 1039-1048.

Mukherjee, K., Brocchieri, L. & Burglin, T.R. 2009. A comprehensive classification and evolutionary analysis of plant homeobox genes. *Molecular Biology & Evolution,* **26(12)**: 2775-2794.

Ori, N., Juarez, M.T., Jackson, D., Yamaguchi, J., Banowetz, G.M. & Hake, S. 1999. Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *knotted*1 under the control of a senescence-activated promoter. *Plant Cell,* **11(6)**: 1073-1080.

Pobudkiewicz, A. 2008. The influence of growth retardants and cytokinins on flowering of ornamental plants. *Acta Agrobotanica,* **61(1)**: 137-141.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y. & Matsuoka, M. 2006. Ectopic expression of *knotted*1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. *Plant Physiology,* **142(1)**: 54-62.

Smith, L.G., Greene, B., Veit, B. & Hake, S. 1992. A dominant mutation in the maize homeobox gene, *knotted*-1, causes its ectopic expression in leaf cells with altered fates. *Development*, **116(1)**: 21-30.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* **3(7)**: 18.

Vollbrecht, E., Reiser, L. & Hake, S. 2000. Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted*. *Development,* **127(14)**: 3161-3172.